

Identification of neutrophil phenotype categories in geriatric hip fracture patients aids in personalized medicine

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Abstract

Objectives: The number of geriatric hip fracture patients is high and expected to rise in the coming years, and many are frail and at risk for adverse outcomes. Early identification of high-risk patients is crucial to balance treatment and optimize outcome, but remains challenging. Previous research in patients with multitrauma suggested that neutrophil phenotype analysis could aid in early identification of high-risk patients. This pilot study investigated the feasibility and clinical value of neutrophil phenotype analysis in geriatric patients with a hip fracture.

Methods: A prospective study was conducted in a regional teaching hospital in the Netherlands. At the emergency department, blood samples were collected from geriatric patients with a hip fracture and analyzed using automated flow cytometry. Flow cytometry data were processed using an automated clustering algorithm. Neutrophil activation data were compared with a healthy control cohort. Neutrophil phenotype categories were assessed based on two-dimensional visual assessment of CD16/CD62L expression.

Results: Blood samples from 45 geriatric patients with a hip fracture were included. Neutrophils showed an increased activation profile and decreased responsiveness to formyl peptides when compared to healthy controls. The neutrophil phenotype of all patients was categorized. The incidence of severe adverse outcome was significantly different between the different categories ($P = 0.0331$). Moreover, patients with neutrophil phenotype category 0 developed no severe adverse outcomes.

Conclusions: Using point-of-care fully automated flow cytometry to analyze the neutrophil compartment in geriatric hip fracture patients is feasible and holds clinical value in determining patients at risk for adverse outcome. This study is a first step toward immuno-based precision medicine for identifying geriatric hip fracture patients that are deemed fit for surgery.

Key Words: geriatric, trauma, hip fracture, neutrophil, inflammatory, flow cytometry

1. Introduction

Geriatric hip fracture patients are a fast-growing and heterogeneous group.^{1,2} Due to an aging population, the absolute number of hip fractures is expected to rise globally to 4.5 million per year by 2050.^{3,4} Many geriatric hip fracture patients are considered frail and, therefore, at risk for adverse outcomes. The 1-year mortality rate after hip fracture surgery is 22%–33%,^{5,6} and the postoperative period after a surgical treatment is associated with a substantial risk of infectious complications such as pneumonia, urinary tract infection, wound infection, and even septic shock.^{7,8} Given the risks of surgery, some patients might be best suited with

nonoperative management. To manage optimal treatment strategies for the geriatric patient with a hip fracture, it is of utmost importance to quickly identify high-risk patients for an adverse outcome. However, a point-of-care clinical parameter to distinguish these patients remains elusive.

Previous research in patients with multitrauma has focused on the immune system for early identification of patients at risk of serious infectious complications. Trauma leads to a complex inflammatory cascade that can cause an acquired immunodeficiency.⁹ It is known that the innate immune system plays an essential role in the defense mechanism against invading

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pathogens.^{10–12} Trauma affects the neutrophil efficacy and therefore makes patients prone to develop infectious complications the days following after trauma.¹² In patients with multi-trauma, a correlation was found between neutrophil phenotype after trauma and the risk of late-onset (>5 days) infectious complications.^{13–17} Recently, it became possible to determine the neutrophil functional phenotype in the acute, point-of-care setting by using an automated flow cytometry approach.¹⁸

Neutrophil phenotype analysis could aid in early identification of frail geriatric patients with a hip fracture. An immunological imbalance could be an early predictor to identify patients at risk for a complicated course which could support the clinician in personalized and shared decision-making. This could have clinical implications because for some patients with very limited life expectancy, nonoperative treatment could also meet their goals of care.^{19,20} The aim of this pilot study was to investigate the feasibility and clinical value of neutrophil phenotype analysis in geriatric patients with a hip fracture in a large regional teaching hospital.

2. Materials and Methods

2.1. Study Design

This prospective study was conducted at a major regional teaching hospital. All geriatric patients aged 70 years or older presenting to the emergency department (ED) of the hospital with a hip fracture from August 1, 2021, to February 1, 2022, were screened for inclusion. Exclusion criteria were as follows: multitrauma (injury severity score [ISS] ≥ 16), transferred from another hospital, no diagnostic blood sampling needed, and a preexistent blood disease. If a patient was eligible for inclusion, blood was drawn and analyzed within 60 minutes.

The medical ethical committee MEC-U, Utrecht, the Netherlands, approved this study under protocol no. R20.054. The study was approved and registered by the Central Committee on Research Involving Human Subjects in the Netherlands under protocol no. NL76875.100.21 and was performed in accordance with the ethical standards established by the Declaration of Helsinki and its later amendments.

2.2. Study Procedure

At presentation, blood was drawn for the standard-of-care geriatric blood panel diagnostic workup. After written informed consent was obtained, one extra 4-mL sodium heparin blood collection tube (Becton Dickinson, Oakville, ON) was drawn specifically for this study. The blood collection tube was placed in the automated AQUIOS CL “Load & Go” Flow Cytometer (Beckman Coulter, Indianapolis, IN), which is located at the ED.

2.3. Healthy Control Cohort

Blood from healthy controls was obtained from healthy individuals participating in the Nijmegen Exercise Study 2021. The blood of the healthy control cohort was drawn on several days before and after a day of a 20–30 km hike. The blood was analyzed by AQUIOS CL using the same protocol as used for the blood of the patient cohort in this study. The median age of the control cohort was 69 years (IQR 66–74). This healthy control cohort was chosen because their relative high age provides a sufficient comparison with the cohort of geriatric patients.

Neutrophils get easily activated by ex vivo manipulation in a time-dependent manner.¹⁸ Therefore, the time between venipuncture and analysis was registered for both the patients and

healthy controls. The healthy control cohort was matched to the patients based on this time until analysis range (analysis within 60 minutes). To rule out time-till-analysis bias, healthy control samples that were analyzed beyond this time frame were excluded. Eventually, 58 healthy control samples could be included in the study.

2.4. Automated Flow Cytometry Analysis

The AQUIOS CL combines automatic sample preparation and flow cytometry analysis of the blood samples. First, the blood collection tube is placed into a cassette into the machine. Next, the machine pipettes the blood into a 96-deep well plate. The blood is then stained for 15 minutes with 18- μ L customized antibody mix for neutrophils. Neutrophil reactivity is tested by analyzing each sample both in the absence and presence of the bacterial/mitochondrial-derived stimulus N-Formyl-norleucyl-leucyl-phenylalanine (fNLF; end concentration 10^{-5} M; BioCat GmbH, Heidelberg, Germany) in the deep well plate. The customized antibody mix contains the following antibodies from Beckman Coulter: CD16-FITC (clone 3G8), CD11b-PE (clone Bear1), CD62L-ECD (clone DREG56), CD10-PC5 (clone ALB1), CD64-PC7 (clone 22). After staining, the red blood cells are lysed by adding 335 μ L AQUIOS Lysing Reagent A (a cyanide-free lytic). The lysis is stopped after 30 seconds by adding 100 μ L AQUIOS Lysing Reagent B, followed by aspiration and analysis through the flow cell.

2.5. Analysis of Flow Cytometry Data

AQUIOS CL flow cytometry data were exported from the device as FCS 3.1 High Res Listmode Files (.lmd). The data were imported and analyzed with an automated clustering (FlowSOM) algorithm on the web-based flow cytometry analysis platform Cytobank (Beckman Coulter, Indianapolis, IN). FlowSOM is a high-dimensional clustering and visualization algorithm, based on a self-organizing maps approach. As described before by Jukema et al,²¹ neutrophils were identified as follows: (1) Granulocytes were gated based on forward/sideward scatter. (2) The granulocytes were analyzed with FlowSOM by using 6 metaclusters and 64 clusters. (3) The neutrophil metacluster was identified by CD16/CD11b expression. For this analysis, all markers of the flowcytometry panel were used (CD10, CD11b, CD16, CD62L, CD64). For each activation marker (CD10, CD11b, CD62L), the median fluorescence intensity (MFI, expressed in arbitrary units) of the neutrophil population, both with and without the addition of fNLF, was exported. Neutrophil reactivity was assessed by calculating a ratio for each activation marker: fNLF-stimulated neutrophil MFI/baseline neutrophil MFI.

2.6. Neutrophil Phenotype Categories

In addition to the .lmd file, a summary .pdf file with two-dimensional dot plots, automatically generated by the flow cytometer, was exported and assessed. All patients were categorized into one of the 7 (0–6) neutrophil immunophenotype categories, as previously described in more detail by de Fraiture et al.¹⁷ These immunophenotype categories are defined based on two-dimensional visual assessment of neutrophil CD16 and CD62L dot plots, from samples analyzed in the absence of fNLF. Category 0 displays a neutrophil receptor expression as seen in healthy control cohorts, while receptor expression on the

neutrophils of patients in categories 1–6 deviates with increased severity of the inflammatory response.

2.7. Clinical Data

The following patient characteristics were collected at baseline: age, sex, trauma mechanism, serum albumin at presentation (g/L), preexistent diagnosis of dementia (from medical records), ASA Physical Status Classification (I to V), treatment (intramedullary osteosynthesis, hemiarthroplasty, total hip arthroplasty, or conservative), and type of hip fracture (femoral neck, intertrochanteric, and subtrochanteric).⁸ Data were collected from the electronic patient record by the treating clinician and anonymously analyzed. Severe adverse outcomes were defined by sepsis and 14-day mortality. Mild infectious complications were definite infections without fulfilling sepsis criteria.

2.8. Statistical Analysis

Baseline characteristics and clinical data were analyzed with SPSS statistical software (version 25.0, IBM Inc. Armonk, NY). Distribution was determined with the Shapiro-Wilk test for normality. Normally distributed continuous data were presented as mean with standard deviation (SD). Non-normally distributed continuous data were presented as median with interquartile range (IQR). The Mann-Whitney *U* test was used to determine statistical differences between 2 groups. The Kruskal-Wallis *H* test was performed to determine overall statistical differences between more than 2 groups. GraphPad Prism (version 8.3.0; Graphpad software, Inc, Sand Diego, CA) was used to analyze and visualize flow cytometry data. A *P*-value of <0.05 was considered statistically significant.

3. Results

In total, 212 patients with a hip fracture presented at the ED between August 1st, 2021, and February 1st, 2022. Of these patients, 48 (23%) were excluded based on the exclusion criteria. This resulted in 164 patients with a hip fracture who were eligible for inclusion. Of these patients, a total of 52 (32%) consented to blood withdrawal for this study. The analysis failed in 5 samples because of human error and in one sample because of a clog in the system of the flow cytometer (Fig. 1). Finally, a total of 46 patients (90% success rate) were successfully analyzed within 60 minutes. One patient was excluded because of a hematological malignancy that interfered with the identification of neutrophils, leaving 45 patients for the final analyses (Fig. 1).

3.1. Baseline Patient Characteristics

The study population consisted of 31 (69%) female patients and 14 male patients with a median age of 82 years (IQR 79–86). All patients sustained a hip fracture after low-energy trauma with ISS <16. Of these patients, 39 (87%) were presented with an isolated hip fracture, whereas 6 patients (13%) had additional injuries after trauma. Baseline characteristics are shown in Table 1.

3.2. Study Population

Forty-two (93%) patients received operative treatment of the hip fracture, and 3 patients (7%) were treated nonoperatively. During hospital admission, 11 (24%) patients developed infectious complications. Of these complications, 8 (18%) were

TABLE 1

Characteristics of Geriatric Hip Fracture Patients

Patient Characteristics	
Age (years)	82 (79–86)
Sex (female %)	31 (69%)
Analyzed samples	52
Successfully analyzed	46
Insufficient blood	2
Wrong barcode on blood collection tube	2
Analyzed without activator agent	1
Clog in the system	1
Time to analysis	
<30 min	41 (89%)
<60 min	5 (11%)
Dementia	9 (20%)
Admission in hospital	44 (98%)
Albumin (g/L)	41.3 (38.9–43.5)
Hypoalbuminemia (<35 g/L)	2 (4%)
Treatment	
Conservatively	3 (7%)
Hemiarthroplasty	22 (49%)
Total hip arthroplasty	5 (11%)
Intramedullary osteosynthesis	14 (31%)
Dynamic hip screw	1 (2%)
Additional injuries	
No additional injury	39 (87%)
Distal radial fracture	2 (4%)
Pubic bone fracture	1 (2%)
Fracture of the olecranon	1 (2%)
Contusio cerebri	2 (4%)
HLOS (days)	6 (4–8)
Infectious complications	11 (24%)
Mild infectious complication	8 (18%)
Sepsis	3 (7%)
14-day mortality	
Yes	3 (7%)
No	42 (93%)
Time from hospital admission to death (days)	16 (3–55)

All variables are in total amount (percentage) or median (IQR).

HLOS = hospital length of stay.

postoperative infectious complications and 3 (7%) consisted of sepsis. The 14-day mortality rate was 7% (3 patients) including the patients who were managed nonoperatively.

3.3. Baseline Neutrophil Activation

Neutrophils of patients with geriatric hip fracture had baseline elevated expression of CD10 (median MFI, 13×10^3 [IQR 11–19 $\times 10^3$] versus 11×10^3 [IQR 8–17 $\times 10^3$], *P* = 0.0133) and CD11b (median MFI, 17×10^4 [IQR 12–24 $\times 10^4$] versus 13×10^4 [IQR 11–16 $\times 10^4$], *P* = 0.0066) compared with healthy controls. Baseline CD62L expression was lower (median MFI, 65×10^4 [IQR 46–72 $\times 10^4$] versus 86×10^4 [IQR 75–96 $\times 10^4$], *P* < 0.0001) in patients with geriatric hip fracture compared with healthy controls (Fig. 2).

3.4. Neutrophil Responsiveness

Neutrophils stimulated with fNLF showed lowered CD10 expression (median MFI, 49×10^3 [IQR 42–58 $\times 10^3$] versus 72×10^3 [IQR 60–89 $\times 10^3$], *P* < 0.0001), lowered CD11b expression (median MFI, 10×10^5 [IQR 8–12 $\times 10^5$] versus 11×10^5 [IQR 10–14 $\times 10^5$], *P* = 0.0007), and decreased CD62L expression (median MFI, 12×10^4 [IQR 7–18 $\times 10^4$] versus $18 \times$

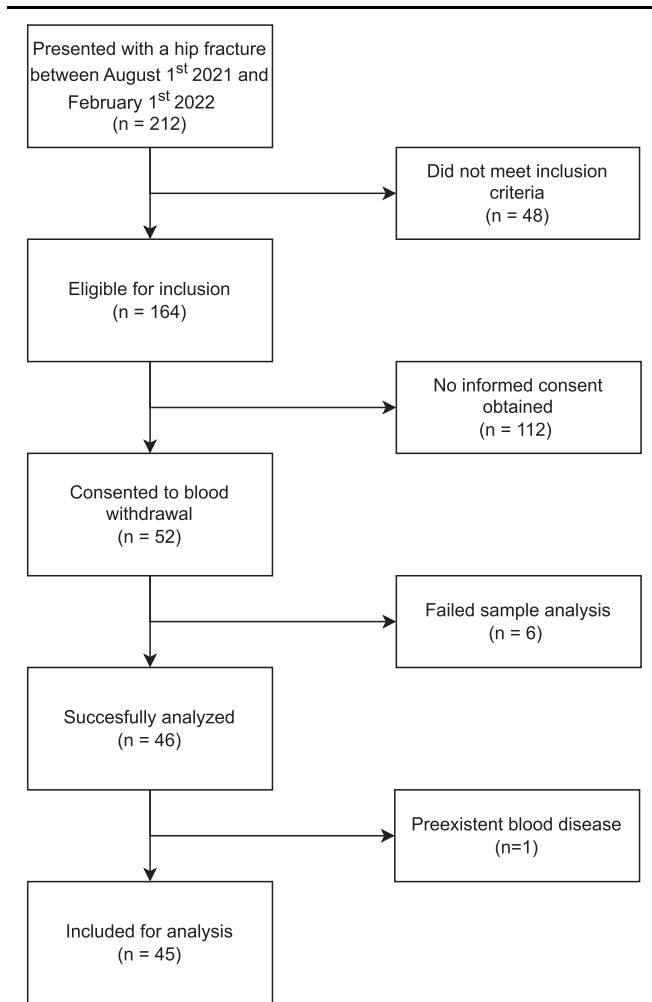


Figure 1. Flowchart of inclusion of patients.

10^4 [IQR 14–23 $\times 10^4$], $P = 0.0002$) when compared with healthy controls.

Neutrophil responsiveness was assessed by calculating a ratio for each marker: fNLF-stimulated neutrophil MFI/baseline neutrophil MFI. Patients with geriatric hip fracture showed reduced neutrophil responsiveness regarding the upregulation of CD10 (median ratio, 3.4 [IQR 2.5–4.0] versus 6.2 [IQR 5.2–7.5], $P < 0.0001$) and CD11b (median ratio, 5.7 [IQR 3.8–7.5] versus 8.7 [IQR 7.5–10.3], $P < 0.0001$) compared with healthy controls. Regarding CD62L downregulation, neutrophil responsiveness was similar for the study cohort and healthy controls.

3.5. Neutrophil Phenotype Categories and Clinical Outcome

Standardized visual assessment of neutrophil phenotype categories identified the presence of 5 of 7 previously described neutrophil subset categories in this study cohort.¹⁷ The categories and the distribution of the patients among the categories are shown in Table 2. Categories 2 and 6 were not present. Of the 12 patients in category 0, one developed an infectious complication and none developed severe adverse outcome. Of the 7 patients in category 1, 2 patients developed an infectious complication and 2 patients died within 14 days. Of the 15 patients in category 3, one patient developed an infectious complication and one patient died within 14 days. Of the 10 patients in category 4, 3 patients developed an

infection and 2 patients developed sepsis. The patient in category 5 developed sepsis and died within 14 days. The incidence of severe adverse outcome (sepsis and/or 14-day mortality) was significantly different ($P = 0.0331$) between the different neutrophil phenotype categories. Neutrophil CD10 and CD11b expression was similar across the different categories (not shown).

4. Discussion

This pilot study aimed to assess the feasibility of identifying patients with geriatric trauma at risk of adverse outcomes after hip surgery by analyzing the functional neutrophil phenotype. The feasibility of Point-of-Care fully automated flow cytometry at the ED to analyze the neutrophil compartment in patients with geriatric hip fracture was demonstrated. This is in line with a previous feasibility study with automated point-of-care flow cytometry in multitrauma population that described a 95% success rate.²² Patients with geriatric hip fracture had distinct neutrophil activation patterns when compared with healthy controls. Furthermore, patients with neutrophil immunophenotype category 0 developed significantly less severe adverse outcome than patients in higher categories.

4.1. Strengths and Weaknesses of the Study

These first of a kind results demonstrate that the use of a point-of-care automated flow cytometer in the trauma geriatric unit is feasible, fast, and reliable. While analyzing the results of this study, a possible manual gating strategy bias was ruled out because the data are analyzed with an automated clustering approach by FlowSOM. Although FlowSOM enables an automated gating strategy, it still requires an extra manual analysis step in a flow analysis program.

Due to the small sample size of this study, the clinical implications of immunophenotype category 1–6 could not yet be appropriately assessed. Earlier research in patients with multitrauma showed that patients who developed infectious complications during hospitalization displayed an extensive presence of neutrophil subsets in the blood immediately after trauma.^{17,22} Further studies in larger geriatric hip fracture cohorts should focus on the role of different neutrophil phenotypes (regarding subsets based on CD16/CD62L expression) immediately after trauma. Second, of 164 patients with a hip fracture who were eligible for inclusion, only 52 patients (32%) gave written informed consent. This was due to frequent delays between diagnostic venipuncture and informed consent procedure causing the requirement of a second venipuncture, which most patients waived. Possibly this could have resulted in some form of selection bias. The use of deferred consent could be a consideration to increase the number of included patients for further research within this field. Moreover, the study population was compared with a healthy control cohort with a lower median age. Although it is highly unlikely that all reported differences are due to this difference is age, it is still preferable that future research with patients with geriatric trauma would be compared with elderly volunteers of a matched age cohort.

4.2. Variety in the Immune Response after Monotrauma in the Geriatric Trauma Patient

In previous research, a very heterogeneous inflammatory response was found in patients with different injury severity.^{17,23} Undoubtedly, among the geriatric hip fracture patients in this

TABLE 2
Infectious Complications and Mortality Across Neutrophil Phenotype Categories in Geriatric Patients After a Hip Fracture

Immunophenotype Category	0	1	2	3	4	5	6	P
N	12	7	0	15	10	1	0	
Infectious complication	N 1	2	—	1	3	0	—	0.0908
Sepsis	N 0	0	—	0	2	1	—	0.0008*
14-day mortality	N 0	2	—	1	0	0	—	0.1421
Severe adverse outcome	N 0	2	—	1	2	1	—	0.0331*

The figures display representative individual samples to illustrate the immunophenotype categories based on the occurrence of subsets of neutrophils in CD16/CD62L dot plots. The patient in Category 5 developed sepsis and died within 14 days. In the other categories, none of the patients developed an infectious complication or sepsis and died within 14 days. Severe adverse outcomes were defined as 14-day mortality and sepsis. P values of the Kruskal-Wallis H test are displayed.

* Significant P values.

study, the amount of tissue damage is lower and more homogeneous than in patients with multitrauma: Almost all patients in this study were presented at the ED after low-energy trauma (fall from stance) resulting in a hip fracture. Nonetheless, a variety of immune activation was found between patients in this cohort. Therefore, it is tempting to speculate that neutrophil activation of the geriatric study population is dependent on a personal immune profile and not so much on the amount of tissue damage as seen in major trauma.

4.3. Neutrophil Activation and Responsiveness

Compared with healthy controls, the patients with geriatric hip fracture showed baseline increased CD10 and CD11b expression, while CD62L expression was lowered, illustrating neutrophil activation.^{21,22,24} The differences observed in baseline neutrophil activation between the study and healthy control cohort could be an effect of aging-related inflammation (so called “inflamm-aging”); a condition that most older

individuals develop and is characterized by elevated levels of blood inflammatory markers, even in absence of active disease.²⁵ Possibly, inflamm-aging would make the neutrophils in the geriatric hip fracture population less responsive to (ex vivo) activation with fNLF because these neutrophils are already activated (primed) by inflammatory markers.¹⁵ The combination of increased neutrophil activation and reduced neutrophil responsiveness demonstrated in the study population can also be caused by hip fracture-related tissue damage: Neutrophils react to tissue damage where they pose a role in tissue regeneration and repair.²⁶

4.4. Neutrophil Immunophenotype Categories

Our results demonstrated that patients in category 0 (receptor expression associated with immune health¹⁷) develop significantly less severe adverse outcomes, when compared with the other categories. This could contribute to clinical decision-making because these patients might be considered fit for surgery,

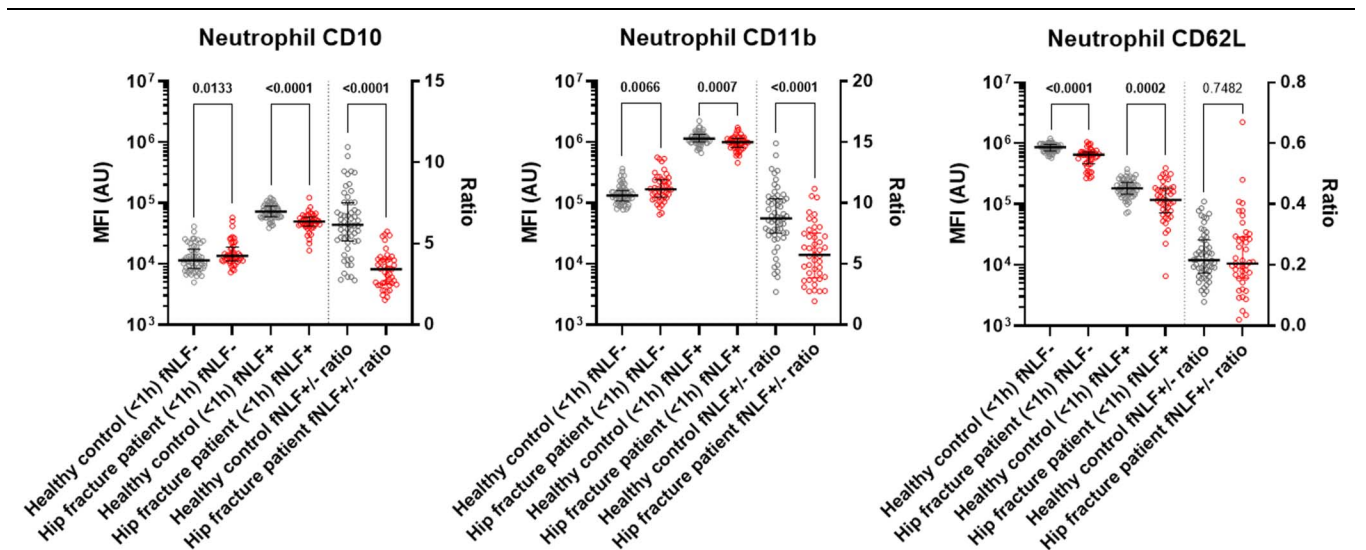


Figure 2. Median fluorescence intensity (MFI) in arbitrary units (AU) of neutrophil activation markers in geriatric patients with hip fracture and healthy controls. Markers are depicted for both unstimulated (fNLF-) and fNLF-stimulated (fNLF+) samples. Neutrophil responsiveness (MFI fNLF+ / MFI fNLF-) is depicted as a ratio for each marker. Statistical significance was tested using the Mann-Whitney U test.

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but this has to be tested in a controlled prospective study. None of the patients displayed category 2 or 6. This is in line with earlier research, where category 2 was only observed in young individuals and category 6 was an extreme immunophenotype that occurs only in patients with extreme injuries, for example, traumatic resuscitation.

4.5. Future Implications

This is the first study that focused on the presence of neutrophil phenotypes in peripheral blood in a geriatric trauma unit. Recent research into shared decision-making in the acute setting emphasizes that palliative, nonoperative management is an acceptable and adequate option for geriatric hip fracture patients with high risk of adverse outcomes after surgery.^{19,27} This study demonstrated that patients with neutrophil phenotype category 0, whom display neutrophil receptor expression is similar as found in healthy controls, develop significantly less severe adverse outcomes, and might thus be considered fit for surgery. However, future research with a larger cohort should further investigate this part of neutrophil phenotyping in geriatric trauma patients.

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